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13. ABSTRACT (Maximum 200 Words)

The etiology of the stress fractyre syndrome is incompetely understood but it is clear that skeletons with high peak bone quantity and quality will be better pretected against incurring this crippling condition. Here, we investigated whether extremely small magnitude (0.3g) but high-frequency (45Hz) mechanical vibrations can alter indices of bone formation, bone resorption, and bone morphology in the growing mouse sksleton. Eight-week-old male BALB/cByJ mice were divided into (1) baseline control, (2) age-matched control, and (3) vibration groups. Vibrations were applied for 15 min/d, either continuously or interrupted by "rest-periods". Mice were sacrificed after either 3wk or 6wk. Three weeks of vibrations applied continuously for 15 min/d decreased osteoclastic bone resorption (Oc.S/BS) by 31% ($p<0.05$) in the trabecular metaphysis and by 33% ($p<0.05$) in the trabecular epiphysis of the tibia while "rest-inserted" loading was ineffective. Low level vibrations applied for 6-wk led to significantly enhanced trabecular bone volume fraction (10%, $p<0.05$) in the tibial epiphysis and thicker trabeculae (5%, $p<0.05$) in the tibial and femoral metaphysis. In summary, an extremely low-level mechanical stimulus, inducing deformations orders of magnitude below those that can actually damage bone, was capable of decreasing bone resorption and enhancing bone morphology – effects that may help to reduce the incidence of stress fractures and to avoid skeletal pathologies later in life.

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Introduction

The stress fracture syndrome is associated with mechanical environments involving very large numbers of loading cycles, yet its etiology is insufficiently understood. It is clear, however, that those recruits with low bone quantity (and bone quality) are not only at greater risk of incurring stress fractures, but are also more likely to suffer from other bone diseases later in life such as osteoporosis. Therefore, interventions which effectively increase bone quantity, and ideally quality, during growth could in turn reduce the incidence of these crippling conditions. FDA approved pharmaceutical interventions of low bone mass may have side-effects and are primarily anti-catabolic in nature. Physical exercise may be capable of positively influencing peak bone mass reached in young adulthood but a principal hurdle has been the identification of safe anabolic and/or anti-catabolic mechanical interventions with high compliance. Mechanical stimuli induced by such interventions would have to be small in magnitude but effective in enhancing peak bone mass and strength to ultimately reduce the incidence of stress fractures and the associated substantial socio-economical costs to the DoD and recruits. In this annual report, we are presenting preliminary data that very low-level mechanical vibrations can effectively reduce bone's catabolic activity and enhance trabecular bone morphology in growing female mice.

Body

Statement of Work #1 -- Anabolic/Anti-Catabolic Assessment of Low Level Mechanical Vibrations in the Growing Mouse Skeleton (3wk protocol)

Experimental Design and Statistics (3wk protocol)

Forty eight-week-old male BALB/cByJ (BALB) mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were randomly divided into four groups (n=10, each group): (1) baseline control (BC), (2) age-matched control (AC), (3) mice vibrated at 45Hz (0.3 g) for 15 min/d, 5 d/wk (MS), and (4) mice vibrated at 45Hz (0.3 g) for 15 min/d, 5 d/wk including "rest-intervals"[Srinivasan et al., 2000], involving the insertion of 10s of "rest" after 1s of loading (MSR). Aside from the "rest-intervals", MS and MSR groups shared the same mechanical parameters including peak accelerations and acceleration patterns, force magnitude, strain frequency, and number of loading cycles per day. Fluorochrome markers (tetracycline, calcein) were injected at specified intervals. After the 3-wk experimental protocol, the right tibia was harvested and fixed in 70% ethanol for micro-computed tomography (μCT) and histomorphometric analyses while the left tibia was fixed in 10% neutral buffered formalin for staining of osteoclastic resorption (tartrate resistant acid phosphatase, TRAP). Experimental procedures were approved by the Institutional Animal Care and Use Committee. All data were expressed as mean ± SD. The difference between BS and AC group was examined by two-tailed t-tests. The difference between MS/MSR and AC group was examined by 2-sided Dunnett t-tests after one-way ANOVA. Statistical significance was set at less than 0.05.

Body Mass

At the commencement of the study, the mean body mass of the four different groups were similar and animals subject to vibration protocols or normal cage activities (age-matched controls) experienced similar changes in body mass over the 3wk period.

Assessment of Osteoclastic Activity (TRAP staining)

Staining of osteoclastic by-products via tartrate resistant acid phosphatase (TRAP) has been described previously [Liu et al., 1987]. The left proximal tibia was fixed in 10% neutral buffered formalin over night, and decalcified in 2.5% formic acid (pH 4.2) for 4 days. Upon dehydration, samples were embedded in glycol methacrylate (GMA) according to the manual of the JB-4 embedding kit (Polysciences, Inc). Frontal sections (7μm) were produced with a microtome and stained for TRAP activity. Hexazotization was achieved by mixing equal amounts of 4% $NaNO_2$ and 4% pararosaniline solution. Naphthol-ASTR-phosphate (Sigma) was used as a substrate, and the enzyme reaction was carried out in the presence of tartrate (10mM) to demonstrate tartrate-resistant bone acid phosphotase activity, at pH 5 in 0.1 M acetate buffer. Sections were counter-stained with methyl green. The ratio of osteoclast surface (Oc.S) to bone surface (BS) was determined for trabecular and cortical bone surfaces in the metaphysis and epiphysis using commercially available histology software (Osteomeasure).

Normal growth within the 3-wk period did not significantly alter activity levels of osteoclasts in the metaphysis of the tibia, but significantly decreased trabecular Oc.S/BS in the epiphysis by 25% ($p<0.01$) (***Figure 1***). Superimposing subtle vibrations onto the skeleton for 15 min/d, however, significantly decreased trabecular Oc.S/BS by 31% ($p<0.01$) in the metaphysis, and by 33% in the epiphysis of tibia ($p<0.01$) (***Figure 1***, ***Figure 2***).

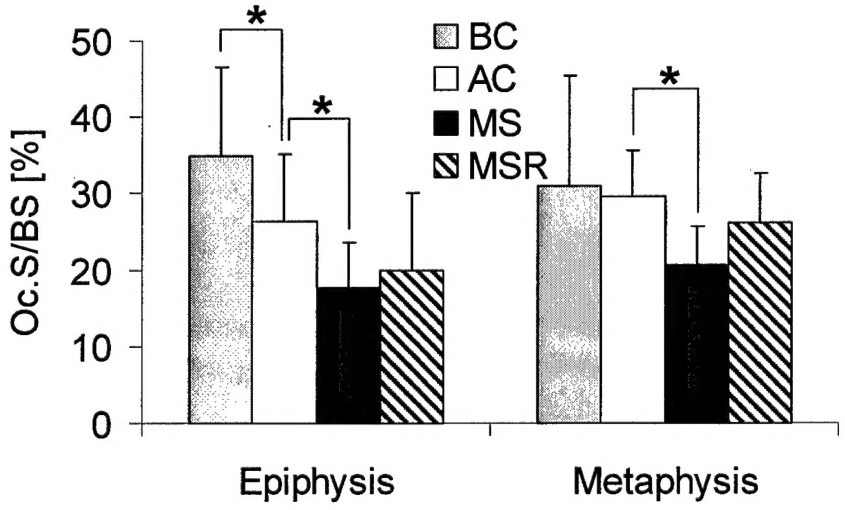

Figure 1. *The percentage of surfaces undergoing osteoclastic resorption (Oc.S/BS) in tibial trabecular bone of the metaphysis and epiphysis. BC = baseline control; AC = age-matched control; MS = mechanical stimululation for 15 min/d; MSR = mechanical stimulation for 15 min/d including rest periods. Asterisks denote statistically significant differences.*

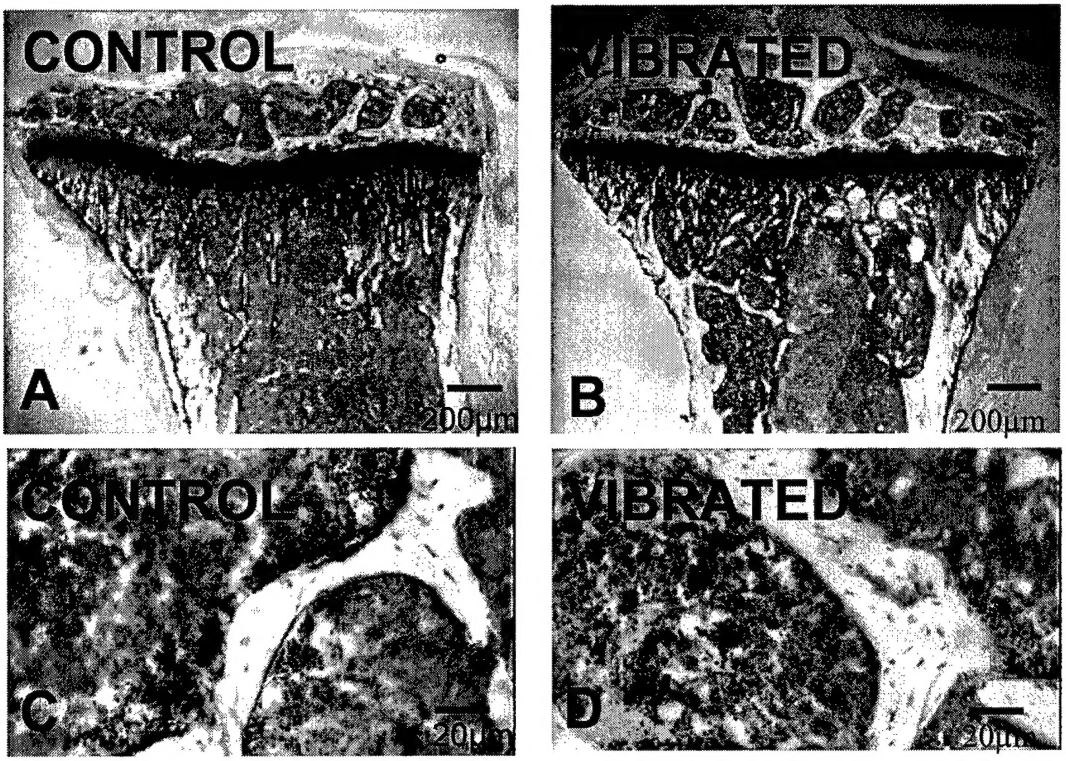

Figure 2. *TRAP staining in the mouse tibia. The red color indicates by-products involved in osteoclastic resorption illustrating that the mechanical intervention significantly reduced resorptive activity. A-B: Proximal tibia (20x). C-D: Proximal metaphysis of the tibia (200x).*

Interestingly, vibrations were able to decrease osteoclastic resorption in both the epiphysis as well as the metaphysis, even though growth-related changes in resorption were very different in these two regions. While osteoclast activity decreased with normal growth in the epiphysis, resorptive activity in the metaphysis did not change over the 3wk experimental period. Thus, low magnitude vibrations were capably of further reducing the decline in osteoclast activity associated with development (epiphysis) as well as to initiate a decrease in resorption (metaphysis). These results indicating that mechanical vibrations can be anti-resorptive are entirely consistent with data from high-intensity exercise [Matsuda et al., 1986], yet at a fraction of the strain and exertion levels that are induced by physical exercise.

Bone Length and Thickness of the Growth Plate

Tibial length was measured with precision digital calipers (Digimatic Caliper; Mitsutoyo, Kanagawa, Japan). The thickness of the growth plate was evaluated using Osteomeasure software. Histomorphometric analysis performed in the growth plate was divided into three different zones: reserved zone, prolifirative zone, and hypertrophic zone. The metaphyseal and epiphyseal borders of the growth plates were defined by the extent of methyl green staining of the cartilage. The hypertrophic-proliferative boundary was defined as the point at which the flattened cell lacunae of the proliferative zone exhibited proximodistal height that was approximately equal to or greater than the transverse dimension [Robling et al., 2001].

Vibrations applied for 15 min/d over a 3 wk period had no detrimental influence on bone length or the thickness of the growth plate and the hyppertrophic zone. These findings emphasize the safe nature of these extremely low-magnitude forces and contrasts with the adverse effects that high-intensity exercise [Matsuda et al., 1986] or large loads at high strain rates (high-impact loading) can have on bone development [Robling et al., 2001].

Statement of Work #2 -- "Long Term" Morphological and Biomechanical Impact of Low Level Mechanical Vibrations on the Growing Mouse Skeleton (6wk protocol)

Experimental Design and Statistics (6wk protocol)
An additional 38 mice were used to assess the long-term (6wk) structural and mechanical consequences of the low-level mechanical intervention. Other than the length of the protocol, the experimental design and statistical analyses used were similar to the 3-wk experimental design described above, also employing baseline control BC (n=8), AC (n=10), MS (n=10), and MSR (n=10) groups. Upon sacrifice, right tibiae and femurs were harvested, cleaned of all surrounding soft tissue and fixed in 70% alcohol for μCT scanning.

Body Mass and Longitudinal Bone Growth
There were no significant difference in body mass or longitudinal growth of the tibia between the control and vibrated groups, inferring that the anti-resoprtive mechanical signal had no significant influence on longitudinal bone growth.

Trabecular and Cortical Bone Morphology (microCT scanning)
The right distal femur and the right tibia were scanned at high (6 μm voxel size) resolution (microCT 40, Scanco Medical). Trabecular bone was separated from cortical bone with manually drawn contour lines. Cortical bone was analyzed from the metaphysis (surrounding the trabecular volume of interest) and from the three diaphyseal regions [Judex et al., 2004a]: the proximal diaphysis (defined at 40% of femoral length), the midshaft (at 50%), and the distal diaphysis (at 60%) (***Figure 3***).

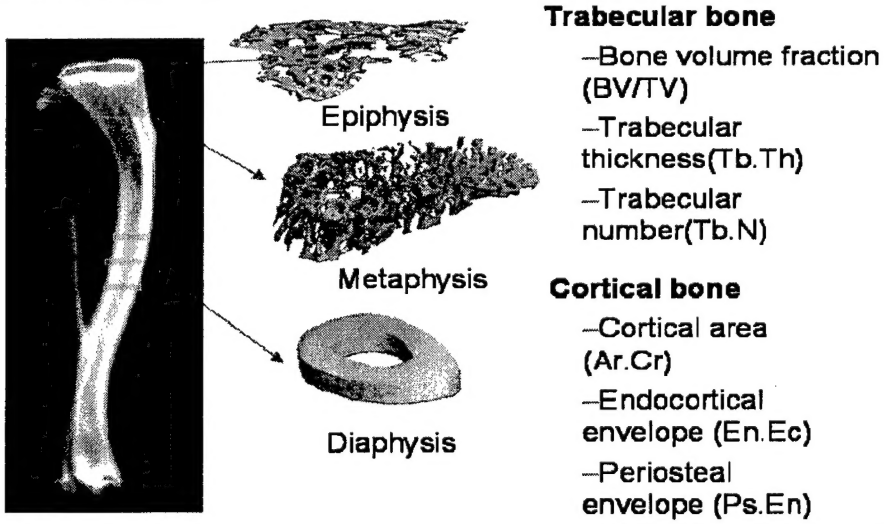

Figure 3. *Five anatomical regions were scanned to detect the effect of low-level vibrations on trabecular and cortical bone morphology in the tibia. Similar regions were scanned in the diaphyseal and distal femur.*

For trabecular bone, bone volume fraction (BV/TV), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), trabecular number (Tb.N), connectivity density (Conn.D), geometrical degree of anisotropy (DA), and the structural model index (SMI) were determined [Judex et al., 2004b]. For all cortical regions, cortical bone area (Ct.Ar) and areas of the endocortical (Ec.En) and periosteal envelopes (Ps.En) were calculated.

After the 6-wk experimental period, trabecular bone volume fraction (BV/TV) in the tibial metaphysis and epiphysis were 60% and 22% ($p<0.01$) greater in age-matched controls when compared to baseline controls (***Figure 4***). Exposing the skeleton to subtle vibrations for 15 min/d without rest led to significantly greater trabecular bone volume fraction (10%, $p<0.05$) in the tibial epiphysis (***Figure 4***), and thicker trabecular (5%, $p<0.05$) in the tibial and femoral metaphysis (***Figure 5***). Vibrations had no significant influence on cortical bone areas or moments of inertia. In contrast to the structural effects induced by continuously applied vibrations, insertion of rest periods rendered the mechanical signal ineffective. These data demonstrate that high-frequency mechanical stimuli at extremely low magnitudes (< 10 microstrain) can site-specifically enhance bone quantity and architecture during skeletal growth without suppressing longitudinal growth.

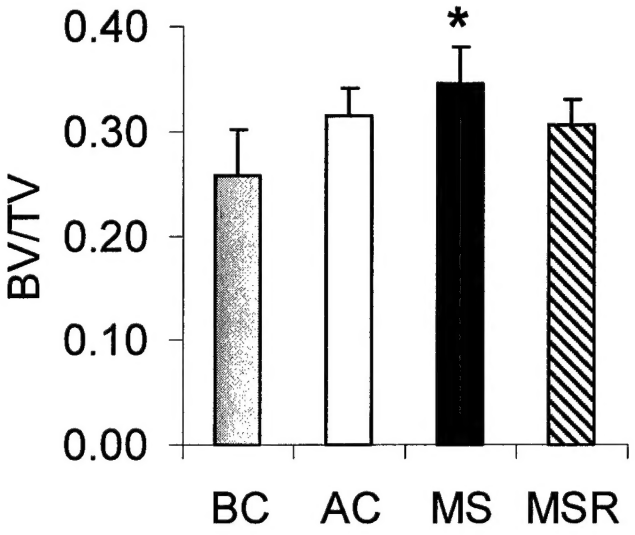

Figure 4. *Bone volume fraction (BV/TV) in the tibial epiphysis in which vibrations applied continuously for 15 min/d significantly enhanced bone morphology (mean+SD, n=10).*

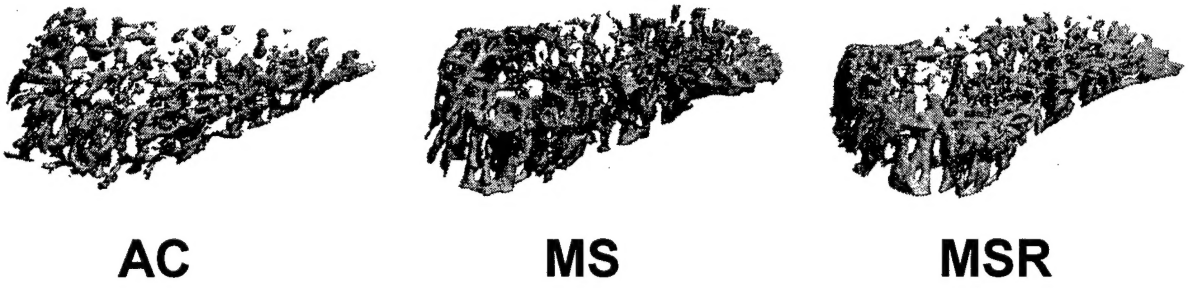

Figure 5. *Three-dimensional micro-CT images of the tibial epiphysis. Trabecular thickness (Tb.Th) was significantly greater in mice subjected to subtle whole body vibrations for 15 min/d (MS) when compared to (mean+SD, n=10). AC = age-matched control; MS = mechanical stimululation for 15 min/d; MSR = mechanical stimulation for 15 min/d including rest periods.*

Mechanical Properties of the Diaphyseal Femur (Four-point Bending)
Four-point bending mechanical testing was used to assess whether vibrations were capable of changing material properties in the cortical diaphysis. Prior to testing, right femurs were soaked over night in PBS at room temperature to ensure full hydration. Mechanical tests were performed using a materials testing system (MTS 858 Mini Bionix II, MTS System Corporation) fitted with a 100 N force cell (Interface, MEG, In Scott Spale, Arizona) transducer. Specimens were loaded in the anterior-posterior plane at a displacement rate of 0.033 mm/s. The distance between the two upper (loading) points was 3.42mm, and the distance between the lower (support) points was 9.6mm. Structural properties were determined from the force-displacement curves: ultimate loading, ultimate displacement, ultimate bending energy, and stiffness.

During the 6-wk experimental period, normal growth substantially altered bone structural properties, however, the application of whole body vibrations did not significantly influence these growth related changes (***Table 1***), entirely consistent with the lack of morphological changes in the cortical diaphysis.

	Ultimate Load (N)	Ultimate Deformation (mm)	Ultimate energy (Nmm)	Stiffness (N/mm)
BS	25.0±5.3	0.58±0.13	7.81±2.65	58.2±15.8
AC	30.2±6.1	0.40±0.09 *	7.45±2.80	77.4±6.12 *
MS	30.8±4.8	0.41±0.08	6.43±1.91	76.2±12.5
MSR	30.0±5.5	0.49±0.11	7.63±2.28	68.1±13.8

Table 1. *Four-point bending of femur of 6-wk experiment. Asterisks denote significant differences between baseline and age-matched controls.*

In summary, these results demonstrate, for the first time, that extremely small levels of vibrations are not only capable of stimulating bone's anabolic activity [Rubin et al., 2001; Judex et al., 2002; Judex et al., 2003], but can also decrease levels of bone resorption. Thus, this biomechanical countermeasure, in contrast to current pharmacologic interventions, may be able to increase peak bone mass and decrease the incidence of osteoporosis later in life by exerting positive effects on both bone formation and resorption.

Key Research Accomplishments

- Low-level (0.3g) whole body vibrations applied for only 15 min/d are anti-resorptive to trabecular bone, structurally enhance bone morphology, and are not detrimental to bone growth or cartilage.
- Changes in bone's cellular metabolic activity induced by low-level mechanical vibrations are modulated by subtle changes in transcriptional levels (Appendix I).
- Low-level mechanical vibrations can induce vascular remodeling in muscle (Appendix II)

Reportable Outcomes

1. Peer-Reviewed Manuscripts Directly Supported by this Grant

Judex, S., Boyd, S., Qin, Y-X, Miller, L., Müller, R., Rubin, C. (2003) Combining high-resolution microct with material composition to define the quality of bone tissue. *Current Osteoporosis Reports* **1**(1). 11-19.

Judex, S., Zhong, N., Squire, M., Garman, R., Ye, K., Donahue, L.R., Hadjiargyrou, M., Rubin, C.T. (in press) Mechanical modulation of molecular signals which regulate anabolic and catabolic activity in bone tissue. *Journal of Cellular Biochemistry.*

2. Conference Proceedings and Abstracts Directly Supported by this Grant

Xie, L.Q., Donahue, L.R., Rubin, C., Judex, S. (2003) Low-level mechanical vibrations enhance bone morphology in the growing skeleton. *Proceedings of the Annual Fall Meeting of the Biomedical Engineering Society*, Nashville, TN.

Garman R, Donahue LR, Rubin C, Judex S. (2004) High-frequency vibrations, in the absence of strain, can increase cortical bone volume. 26th Annual Meeting of the American Society of Bone and Mineral Research, Seattle, WA. *Journal of Bone and Mineral Research **19**(S1), p. 258.*

Choi, E., Judex, S. (2004) The identification of tartrate resistant acid phosphatase as a quantitative assessment of bone resorption. *Proceedings from the 2004 URECA Celebration.* Stony Brook, NY. p. 81.

Murfee, W.L., Hammett, L.A., Xie, L.Q., Squire, M., Judex, S., Skalak, T.C. (2004) High-frequency low-magnitude vibrations may induce vascular remodeling in skeletal muscle. *Proceedings of the Annual Fall Meeting of the Biomedical Engineering Society*, Philadelphia, PA.

Xie, L., Choi, E., Donahue, L., Rubin, C., Judex, S. (2004) Low-level mechanical vibrations reduce bone resorption in the growing skeleton. 26th Annual Meeting of the American Society of Bone and Mineral Research, Seattle, WA. *Journal of Bone and Mineral Research **19**(S1), p. 397.*

Conclusions

The anabolic effect of extremely low-level whole body vibrations has been previously demonstrated in the adult skeleton of a variety of species. Our current data indicate that these signals may not only be anabolic but may also be anti-resorptive in the growing skeleton. The extremely small deformation magnitudes induced in the bone matrix, together with the short duration at which these signals become effective (15 min/d in this study) suggest that vibrations may be a safe and effective intervention to increase peak bone mass – thereby, perhaps, reducing the incidence of stress fractures in army recruits and preventing the occurrence of bone diseases (e.g., osteoporosis) later in life.

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Appendices

1. Judex, S., Zhong, N., Squire, M., Garman, R., Ye, K., Donahue, L.R., Hadjiargyrou, M., Rubin, C.T. (in press) Mechanical modulation of molecular signals which regulate anabolic and catabolic activity in bone tissue. *Journal of Cellular Biochemistry.*
2. Murfee, W.L., Hammett, L.A., Xie, L.Q., Squire, M., Judex, S., Skalak, T.C. (2004) High-frequency low-magnitude vibrations may induce vascular remodeling in skeletal muscle. *Proceedings of the Annual Fall Meeting of the Biomedical Engineering Society,* Philadelphia, PA.

MECHANICAL MODULATION OF MOLECULAR SIGNALS WHICH REGULATE ANABOLIC AND CATABOLIC ACTIVITY IN BONE TISSUE

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ABSTRACT

Identifying the molecular mechanisms that regulate bone's adaptive response to alterations in load bearing may potentiate the discovery of interventions to curb osteoporosis. Adult female mice (BALB/cByJ) were subjected to catabolic (disuse) and anabolic (45 Hz, 0.3g vibration for 10 min/d) signals, and changes in the mRNA levels of thirteen genes were compared to altered indices of bone formation. Age-matched mice served as controls. Following four days of disuse, significant ($p \leq 0.05$) decreases in mRNA levels were measured for several genes, including collagen type-I (-55%), osteonectin (-44%), Osterix (-36%) and MMP-2 (-36%) all of which, after 21d, had normalized to control levels. In contrast, expression of several genes in the vibrated group, which failed to show significant changes at four days, demonstrated significant increases after 21d, including iNOS (39%, $p=0.07$), MMP-2 (54%), and RANKL (32%). Correlations of gene expression patterns across experimental conditions and time-points allowed the functional clustering of responsive genes into two distinct groups. Each cluster's specific regulatory role (formation vs resorption) was reinforced by the -60% suppression of formation rates caused by disuse and the 55% increase in formation rates stimulated by mechanical signals ($p<0.05$). These data confirm the complexity of the bone remodeling process, both in terms of the number of genes involved, their interaction and coordination of resorptive and formative activity, and the temporal sensitivity of the processes. More detailed spatial and temporal correlations between altered mRNA levels and tissue plasticity may further delineate the molecules responsible for the control of bone mass and morphology.

Keywords: Bone Adaptation, Mechanical Loading, Disuse, Gene Expression, Osteoporosis

INTRODUCTION

Functional load bearing is a strong influence on bone remodeling, as evidenced by its direct impact on osteoblastic and osteoclastic activity, and ultimately, by alterations of bone mass and morphology. Decrease in functional loading, which occurs under conditions such as spaceflight or bedrest, suppresses osteoblast activity while elevating osteoclastic activity [Turner, 2000], resulting in a net loss of bone [Alexandre et al., 1988; Judex et al., 2004]. Conversely, specific increases in load bearing, as in the form of exercise, can stimulate osteoblastic activity while suppressing osteoclast recruitment and activity [Judex et al., 2000; Tajima et al., 2000], resulting in net improvements in both bone quantity and quality [Rubin et al., 2002; Judex et al., 2003].

In vivo studies which have investigated the influence of mechanical loading on bone plasticity have focused on identifying those specific mechanical parameters responsible for regulating the response. By design, many of these studies have precluded a rigorous characterization of the molecular mechanisms which control bone's adaptive processes, thus hampering a full understanding of the etiology of osteoporosis, and limiting a systematic examination of potential drug targets involved in regulating bone disease. *In vitro* models of bone adaptation have been used to demonstrate that a number of diverse molecules are related to mechanically mediated changes in bone formation and resorption – ranging from immediate early genes such as c-fos to the most abundant protein in bone, collagen type I [Jiang et al., 2002; Saunders et al., 2003; Peake et al., 2003; Hatton et al., 2003; Genever et al., 2001; Rubin et al., 2000; Nomura et al., 2000]. Critical putative functions of genes involved in mechano-reception and transduction have been derived from these studies. Ultimately, however, removal of cells from their matrix may mask critical interdependent interactions between osteoblasts, osteoclasts, osteocytes and other cells [Parfitt, 1995], and emphasizes the benefits of using integrative *in vivo* models to more fully characterize the molecules which ultimately control bone remodeling activity, and more rigorously consider the complexity of the bone remodeling response [Karsenty, 2003; Hadjiargyrou et al., 2002; Pavlin et al., 2001].

We have recently demonstrated that extremely small magnitude forces, induced non-invasively to the skeleton as whole body vibrations, can be perceived as osteogenic [Rubin et al., 2001a]. In the proximal tibia of adult BALB/cByJ (BALB) mice, for example, 10 minutes per day of a 45 Hz, 0.3g acceleration (1g = acceleration on Earth, or $9.8 m/s^2$) is anabolic to trabecular bone [Judex et al., 2002], while disuse is catabolic [Judex et al., 2004] and suppresses bone formation [Judex et al., 2002]. The goal of the study reported here was to relate these mechanically mediated changes in bone formation rates to the expression of a broad set of genes anticipated to play a role in regulating bone adaptation. Thirteen genes were considered, all of which may serve critical, but not necessarily unique, roles in either the formative (Cbfa1 [Ontiveros et al., 2003], osterix [Nakashima et al., 2002] , BMP-2 [Sato et al., 1999], IGF-1 [Kawata et al., 1998], MMP-2 [Blumenfeld et al., 2002], collagen type I [Moalli et al., 2000], integrin β3 [Weyts et al., 2002], osteonectin [Pioletti et al., 2003]) and resorptive (RANKL [Rubin et al., 2003], iNOS [Watanuki et al., 2002], osteopontin [You et al., 2001], MMP-9 [Rantakokko et al., 1999], cathepsin K [Rantakokko et al., 1999]) events of (mechanically induced bone) remodeling. It was hypothesized that alterations in load bearing (increase or decrease) will stimulate differential responses in the activity of these formation and resorption gene "families," including their temporal expression patterns.

METHODS

Experimental Design

All procedures were reviewed and approved by the University's Animal Care and Use Committee, and met or exceeded all AALAC guidelines. Sixteen-week old female BALB/cByJ mice were randomly distributed into one of three groups: control, disuse, and mechanical stimulation (n=9, each). Mice were housed individually in standard cages (28*x*17*x*13 cm) and had access to rodent chow (autoclaved diet NIH-31 with 6% fat, 18% protein, Ca:P 1:1, vitamin and mineral fortified) *ad libitum*.

The catabolic stimulus was achieved by functional disuse, initiated by applying (24 h/d) a hindlimb unloading apparatus (tail suspension), originally developed for the rat [Morey-Holton et al., 2002], and modified for use in the mouse [Judex et al., 2002]. The mechanically mediated anabolic signal was achieved by placing them on a platform which oscillated vertically at 45Hz, 0.3g, for 10 min/d, 5 d/wk [Judex et al., 2002]. Six mice from each group were sacrificed after 4d, and nine mice from each group sacrificed after 21d. To facilitate the accurate measurement of bone formation, mice in the 21d group were injected with calcein (i.p.,10 mg/kg) on days 10 and 19. Upon sacrifice, left and right tibiae were harvested, cleaned of all surrounding soft tissue, and preserved in 70% EtOH (right tibia; histomorphometry), or immediately snap-frozen in liquid nitrogen and stored in -80°C (left tibia; gene expression).

Histomorphometry

Processing of the mouse tibia for histomorphometry has been described previously [Judex et al., 2002]. Briefly, the proximal and diaphyseal region of the right tibia of each mouse was embedded in methyl-methacrylate (Fisher Scientific, NJ). Following polymerization, 7-μm-thick undecalcified frontal section was cut with a sledge microtome (Reichert Jung, Germany) from the proximal tibia and a 40 μm transverse section was cut from the middiaphysis with a diamond wire saw (Well Diamond Wire Saws, GA). For each tibia, mididaphyseal cortical (Ct.Ar) and metaphyseal trabecular bone area (Tb.Ar) were measured and bone formation rates (BFR) were determined with bone surface (BS) as referent. Further, mineralizing surface (MS/BS), an indicator for the presence of osteoblasts on surfaces and mineral apposition rates (MAR), an indicator of how fast osteoblasts are producing new bone, were measured. All analyses were performed with the operator blinded to the identity of the mouse from which the section had been harvested. Bone sections that failed to display double labels anywhere within the entire tibial section, indicating that one of the fluorescent label injections was not

absorbed, were removed from the dynamic bone analyses (applicable to one specimen from the control group). To compare changes in gene expression (based on RNA extracted from the entire tibia) to altered static and dynamic histomorphometric bone indices at the tissue level, all formation rates and bone areas were averaged between trabecular and cortical bone.

RNA Extraction

Upon storage in -80°C, individual left tibiae (including bone marrow and cartilage) were crushed into a fine powder using a liquid nitrogen cooled mortar and pestle, and total RNA was isolated using standard protocols [Reno et al., 1997; Majima et al., 2000]. TRIzol reagent (Life Technologies, MD) was added to the bone powder and further homogenized. Phases were separated with chloroform under centrifugation (12,000g). One volume of ethanol was added to the aqueous phase and total RNA was isolated using the RNeasy Total RNA isolation kit (Qiagen, CA) as directed by the manufacturer. Any potential DNA contamination was removed with RNase free DNase (Qiagen). A cyto-fluorometer and a fluorescent dye (Ribo Green, Molecular Probes, OR) were used to quantify the amount of total RNA extracted from the tibia. The integrity of all RNA samples was assessed by agarose gels and RT-PCR amplification curves, leading to the exclusion of three bones from each of the 21d groups. Consideration of a relatively large number of genes across three different experimental groups (control, vibrated, disuse) and two time points (4d & 21d) necessitated the pooling of RNA samples [Kendziorski et al., 2003] for each group at a given time point (2 pools of n=3). Experimental error was minimized by running all samples (pools) in triplicates, and the average of the three values was used for further analyses.

Quantification of Gene Expression

The thirteen candidate genes monitored in this study were selected as based on their proposed critical roles in regulating bone remodeling activity. Although most, if not all, of the chosen genes

almost certainly have multiple functions, the 13 candidate genes were categorized into those critical to: 1) formative processes, and 2) resorptive processes. Of course, the pool of possible candidate genes implicated in bone formation and resorption is enormous [Karsenty, 2003; Teitelbaum et al., 2003] and an entirely different set of candidate genes could have been used to characterize changes in the mechanically mediated expression levels of formative and resorptive genes as a function of time. Thus, many genes essential to the control of bone's response to mechanical perturbations were inherently not considered. Similarly, the number of possible time points, especially for the screening of immediate early genes, is essentially infinite, and the 4d and 21d time points were chosen as indicators of relatively early and intermediate processes occurring at the tissue level [Pead et al., 1988].

Genes characterized as essential to bone formation included: core binding factor alpha-1 (Cbfa1), osterix, insulin like growth factor-1 (IGF-1), bone morphogenetic protein 2 (BMP-2), integrin β3, collagen type I (α1), metalloproteinase-2 (MMP-2 or gelatinase A), and osteonectin. Genes related to bone resorption included: receptor activator of the nuclear factor kB ligand (RANKL), inducible nitric oxide synthase (iNOS or NOS2), cathepsin K, metalloproteinase-9 (MMP-9 or gelatinase B), and osteopontin. The expression levels of these 13 candidate genes were quantified using a real-time PCR cycler (LightCycler, Roche, IN) relative to the expression levels of a housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH).

Mouse specific primers for the coding region of each gene were designed (LightCycler primer design software) and verified through sequencing. A "one-step" real-time RT-PCR kit (Qiagen, CA) provided the chemistry for the quantification process (e.g, reverse transcriptase, buffer, nucleotides, SYBR Green). Annealing temperature and the amount of RNA template (~ 1-10 ng) were optimized for each primer pair. The cycling conditions for each reaction consisted of reverse transcription at 50°C for 20 min, activation at 95°C for 15 min, and amplification for 40 cycles. Each amplification cycle included denaturation at 94°C for 15 sec, annealing at temperatures specified in **Table 1** for 20

seconds, and extension at 72°C for 12 seconds. Upon amplification, a melting curve analysis (95°C for 5 sec, 55°C for 15 sec, and 95°C for 0 sec) was performed to check for single amplicons. Both the gene of interest and the housekeeping gene were included in the same RT-PCR run to minimize variations. Each run also included standard curves for both genes using serially diluted RNA from a generic mouse tibia. LightCycler software quantitated the expression level of the gene of interest with GAPDH expression levels as a referent.

Statistical Analyses

T-tests with Bonferroni corrections for multiple comparisons conservatively tested for differences in histomorphometric indices between the control and the two experimental groups. Temporal changes in body mass within a group of mice were assessed by paired t-tests.

The pooling of RNA from three animals within each group (run as triplicates) afforded t-tests (with conservative Bonferroni corrections) to compare gene expression levels between experimental (vibrated or disuse) and control mice [Kendziorski et al., 2003]. For a given gene, the true expression level in animal j of group i can be written as $y_{ij} = \mu_i + \varepsilon_j$, where μ_i is the group mean and ε_j represents the biological variation between individual animals. The k^{th} measurement (measurements were made in triplicates) of the j^{th} pool (of three animals) in the i^{th} group can be written as:

$$z_{ijk} = \mu_i + \frac{\varepsilon_{j_1} + \varepsilon_{j_2} + \varepsilon_{j_3}}{3} + \delta_k,$$

where δ_k is the measurement error. The T-test statistic to determine the difference between the control and experimental groups becomes $T = \frac{\overline{Y}_1 - \overline{Y}_2}{S.E.(\overline{Y}_1 - \overline{Y}_2)}$, where $\overline{Y}_i$ is the average of two pools in each group, and the denominator is the standard error of the numerator. Therefore, the variance of $\overline{Y}_1 - \overline{Y}_2$ equals $1/3*(\sigma^2_{\text{biologic}} + \sigma^2_{\text{measurement}})$, where $\sigma^2_{\text{biologic}}$ is the variance of the biological variation ε

(reflected in the variation between the two pools of three animals each) and $\sigma^2_{measurement}$ is the variance of the measurement errors δ (reflected in the variation between the three measurements for each pool). Assuming $\sigma^2_{biologic}$ and $\sigma^2_{measurement}$ are similar for all six groups (3 groups*2 time points) for a given gene (i.e, the underlying distribution for the population from which the two pools were drawn is similar in shape for all groups), the standard error $1/3*(\sigma^2_{biologic} + \sigma^2_{measurement})$ is estimated by $\sum_{i=1}^{6}(\overline{Z}_{i1} - \overline{Z}_{i2})^2 / 12$ where i is the index of the six groups from the two time points and $\overline{Z}_{i1}$ and $\overline{Z}_{i2}$ are the average measurements of pools 1 and 2 . Therefore, the t-statistic to compare the control group to an experimental group was computed as:

$$T = \frac{\overline{Y}_{control} - \overline{Y}_{exp}}{\sqrt{\sum_{i=1}^{6}(\overline{Z}_{i1} - \overline{Z}_{i2})^2 / 12}}.$$

The degrees of freedom for the estimate of the standard error was six and p-values were calculated based on the underlying student-*t* distributions.

To contrast alterations in gene expression between the two time points, a t-statistic similarly to the one above was obtained. The numerator is the difference between the experimental and control groups across the 4d and 21d time points and the denominator is its standard error, i.e. $\sqrt{\sum_{i=1}^{6}(\overline{Z}_{i1} - \overline{Z}_{i2})^2 / 6}$. Corresponding p-values were also determined with 6 degrees of freedom. See [Kendziorski et al., 2003] for a more detailed discussion on using statistical tests for pooled samples.

Linear correlations tested the association of gene expression patterns across the three conditions and two time points. To avoid mapping of potential "noise", only genes that were either (1) differentially regulated at least at one time point (or treatment) or (2) exceeded changes in mRNA levels by 10% at least at two time points (or treatments) were include in this analysis. Expression patterns of genes which were highly correlated to each other (R>0.6) were grouped into clusters.

Multi-factorial univariate ANOVA tested for main effects and interactions of the two fixed factors, (1) time point and (2) variations in the induced mechanical environment. Differences in the latter factor were followed up with Tukey post-hoc tests. All tests were run separately for genes that had been categorized as bone formation or bone resorption genes. Histomorphometric data were presented as mean ± SD and gene expression data were presented as averages of the three runs for each pool. Statistical significance was set at p=0.05 and p-values between 0.05-0.07 were considered as borderline significant.

RESULTS

Body Mass and Histomorphometry

No significant differences in body mass were detected between the three experimental groups at baseline, at 4d, or at 21d, although disuse mice lost 4% (p=0.02) of their weight over the 21d protocol (no significant changes were observed in the other two groups).

Bone formation rates in the tibial metaphysis (trabecular bone) and the perosteal and endocortical surfaces of the diaphysis (cortical bone) were measured in the 21d animals to characterize the tissue level response of the tibia to alterations in its normal functional environment. Averaged across trabecular and cortical bone, bone formation rates (BFR/BS) were 55% (p=0.04) greater in mice exposed to the low-level mechanical signal and 60% (p=0.002) lower in disuse mice when compared to control mice (**Figure 1**).

Changes in bone formation rates were relatively uniform across the trabecular and cortical surfaces. In trabecular bone, vibration stimulated a 32% (p=0.03) increase in BFR while removal of functional load bearing caused a 55% (p=0.001) reduction in BFR. In diaphyseal cortical bone, BFR were 93% (p=0.12) greater at the periosteal surface (62%, p>0.05, at the endocortical surface) of mice subject to 10 min/d of vibration than in control mice. In contrast, disuse caused a 60% (p=0.04)

decrease in BFR at the periosteal surface (69%, p=0.02, at the endocortical surface). Ten minutes of daily vibrations up-regulated mineralizing surface (MS/BS) in trabecular bone (18%, p=0.04) and mineral apposition rates (MAR) at the periosteal surface (45%, p=0.05) while disuse down-regulated MS/BS (-20%, p=0.05) and MAR (-45%, p=0.001) in trabecular bone, MS/BS at the periosteal surface (-63%, p=0.01), as well as MS/BS (-49%, p=0.03) and MAR (-37%, p=0.02) at the endocortical surface of the middiaphysis. Exposing the tibia to altered levels of mechanical load affected bone volume only in the trabecular metaphysis of disuse mice which decreased by 43% (p=0.003).

Gene Expression

Four days of disuse caused the expression of several genes involved in bone formation to be down-regulated, including: osterix (-36%, p=0.001), BMP-2 (-26%, p=0.06), collagen type I (-55%, p=0.003), osteonectin (-44%, p=0.02), and MMP-2 (-36%, p=0.05) (**Figure 2**). Of the candidate genes proposed to be critical to bone resorption, osteopontin was up-regulated (+30%, p=0.07) (**Figure 3**). After 21d of disuse, the expression levels of all genes, except osterix, had reestablished the levels measured in age-matched control mice. The expression of genes coding for integrin-β3, Cbfa1, RANKL, cathepsin-K, and MMP-9 were not significantly affected by disuse at either the 4d or 21d time-point (Table **3**).

Four days of mechanical stimulation failed to affect the mRNA levels of any of the candidate genes when compared to controls. In contrast, following 21d of mechanical stimulation, larger changes in gene expression were observed. MMP-2 mRNA levels were significantly up-regulated by 54% (p=0.03) while osterix (+14%, p=0.17), BMP-2 (+18%, p=0.22), and collagen type I (+19%, p=0.20) levels were modestly, but not significantly, altered. Representing genes from the "resorption" group, RANKL (+32%, p=0.05) and iNos (+ 39%, p=0.07) were significantly up-regulated or were borderline significant (Figure **3**).

Using those nine genes which expression values were either affected by vibration and/or disuse (MMP-2, Osteonectin, osterix, BMP-2, coll-I, iNOS, osteopontin, RANKL) or exceeded changes of 10% over at least two time points (Cbfa1), linear correlations (across the experimental treatments and time points) associated genes with each other such that genes with high correlation coefficients were grouped into the same cluster. Two clusters were sufficient to generate correlation coefficients above 0.6 for each gene, except RANKL. As hypothesized, genes that were anticipated to functionally relate to each other generally fell into the same cluster. Cluster 1 contained primarily bone formation genes, including Cbfa1, osterix, BMP-2, osteonectin, MMP-2, and collagen type-1 (**Table 4**). iNOS and osteopontin defined cluster 2 (R=0.68). RANKL did not fit into any either cluster; the only gene which expression pattern produced a correlation coefficient of above 0.35 with RANKL was iNOS (R=0.52).

For further analysis, a multifactorial analysis of variance (including post-hoc test) was used for the same nine genes that had been grouped according to their putative anabolic (Cbfa1, osterix, BMP-2, osteonectin, MMP-2, and coll-1) or catabolic (RANKL, osteopontin, iNOS) character, testing for main effects and interactions between the two factors used in this study: (1) *time point* [4d, 21d] and (2) *variations in the induced mechanical environment* [control, vibration, disuse]. Again, the assignment of genes to either the anabolic or catabolic category was not unique; however, several permutations in gene assignment were tested and did not substantially alter the results. The different levels of applied mechanical stimuli significantly ($p<0.001$) affected the mRNA levels of bone forming genes, with both vibration ($p=0.03$) and disuse ($p<0.001$) being different from control. The significant ($p<0.001$) interaction between time and the level of mechanical use (i.e., no independence between these two variables) reflected the inverse temporal expression patterns for mechanical stimulation and disuse (mRNA levels were altered by disuse primarily after 4d and by mechanical stimulation after 21d). Similarly, the level of mechanical load ($p=0.04$) and the interaction between load and time ($p=0.01$) had significant effects on the three bone resorptive genes, but significant differences in gene

expression values across the two time points could only be established between control and vibrated mice.

DISCUSSION

The work presented here supports the premise that bone is sensitive to both increases and decreases in mechanical loading, and that the altered remodeling caused by such perturbations (enhanced bone formation by low-level mechanical signals, suppressed bone formation by disuse) is regulated, ultimately, by a complex, temporally dependent, interaction of a multitude of genes. Changes in gene expression stimulated by extremely low-level mechanical signals contrasted sharply with altered gene expression induced by disuse, and in both cases, the gene activity was dependent on time. The molecular events controlling the adaptive response in bone, as indicated by relative changes in expression patterns of several candidate genes coding for transcription factors, cytokines, proteins, or proteases, were distinct for increased and decreased levels of mechanical loading. While thirteen genes were examined, following these studies it was clear that the responsive genes could be functionally grouped into two clusters; those whose primary function has been primarily linked to bone formation and those whose expression activity has been largely linked to bone resorption.

Disuse had its predominant effect on gene expression patterns early on, as reflected by the increases and decreases measured at day 4, but the anabolic signal did not influence the candidate gene pool until much later. Certainly, there is a strong likelihood that a broader pool of genes would reveal some that were perturbed by the anabolic process earlier than three weeks, particularly considering the morphologic changes that are observed in bone lining cells following brief bouts of loading [Chow et al., 1998]. However, it was surprising that – even with a mechanical signal that stimulated as much as a 90% increase in bone formation rates, that none of the selected genes were influenced early on and certainly implies that other genes are intricately involved in the adaptive process at these early time points. Alternatively, large expression changes in small regions within the bone may have been

masked because of surrounding non-responsive regions – despite the spatial similarities in altered bone formation rates between trabecular and cortical surfaces. Further, pooling of RNA samples reduced statistical power and future detection of small (but significant) changes in mRNA levels will be aided by larger sample sizes.

Serum levels of molecules important for bone metabolism (e.g., calcium, PTH) were not measured in this study. While it is clear that altered circulating levels of systemic factors can affect gene expression levels, this study was performed *in vivo* to obtain the integrative (direct and indirect) adaptive changes in mRNA levels in a physiological system. Nevertheless, future correlations of altered gene expression levels with altered serum molecules will yield important data on the relative contributions of direct and indirect effects of anabolic and catabolic signals in bone. Further, assays to estimate changes in bone resorptive activity (e.g., TRAP staining for osteoclast products) was not addressed, and thus a direct link between the "bone resorption genes" and osteoclast activity could not be assessed. However, the large reduction in bone volume induced by disuse suggests that disuse, *per se,* not only suppressed bone formation activity, but elevated resorptive activity [Beamer et al., 1996]. Of course, the subset of genes which increased their activity with disuse might better be explained by a functional role in the recruitment and differentiation of osteoclasts rather than an active role in the suppression of formation.

None of the 13 candidate genes monitored at either time point were simultaneously affected by the catabolic and anabolic mechanical perturbations. This indicates that the functional role of the candidate genes is specific, and may be interpreted to indicate that the processes responsible for the increase in bone formation activity are independent from processes that are related to the suppression of bone formation activity. Given the large, consistent with previous investigations [Rubin et al., 2001b; Bikle et al., 2003; Oxlund et al., 2003], increases and decreases in mechanically mediated bone formation rates, it is striking that the changes in gene expression were not particularly robust (< 2-fold), indicating that while the molecular regulation of bone adaptation is exceedingly complex, it is

also very subtle – particularly when compared to the much larger events such as bone repair [Hadjiargyrou et al., 2002] or the regulation of development and growth [Sodek et al., 1995; Enomoto-Iwamoto et al., 2002].

Collagen type-I, the most abundant matrix protein in bone, displayed the most predictable [Ahdjoudj et al., 2002] expression pattern following the perturbation of mechanical signals. The 55% reduction in activity following just 4d of disuse closely paralleled the mean 60% reduction in formation rates in trabecular and cortical bone. That collagen expression had normalized back to control levels following 21d of disuse should not be completely surprising, given that the dynamics of changes in bone formation due to disuse is somewhat transient in nature [Globus et al., 1986]. In direct contrast with disuse, mechanical stimulation failed to mobilize collagen expression at 4d, but expression patterns were moderately up-regulated at 21d. This delay, too, is not surprising considering the time it may take for the mechanical signal to recruit (and differentiate) bone cell populations such as bone lining or osteo-progenitor cells into osteoid producing osteoblasts [Chow et al., 1998].

The transcription factors Cbfa1 and osterix are essential for inducing osteoblast differentiation [Ducy et al., 1997; Nakashima et al., 2002; Otto et al., 1997]. Surprisingly, despite the distinct changes in bone formation rates caused by mechanical stimulation and disuse, neither perturbation altered mRNA levels of Cbfa1. In contrast, disuse resulted in a rapid and persistent down-regulation of osterix. Osterix, which acts downstream of Cbfa1, is regulated by several growth factors, including bone morphogenetic proteins (BMPs) that also play critical roles in osteoblast differentiation [Sakou, 1998]. The similar expression patterns between BMP-2, Cbfa1, osterix, and collagen type I in response to the anabolic and catabolic signals across the two time points helped to include them all in functional Cluster 1, and suggests that these genes are critical to the tight up- and down regulation of bone's anabolic activity perturbed by positive and negative mechanical events.

Demonstrating similar patterns of expression [Lockhart et al., 2000], two other genes, osteonectin and MMP-2, were found in this same functional cluster, and perhaps perform similar functions in the

control of the cascade of adaptive processes. Osteonectin is a non-collagenous bone protein involved in regulatory interactions between bone cells and the extracellular matrix [Bradshaw et al., 2001]. Genes such as osteonectin are considered critical for maintaining both bone quantity and quality [Delany et al., 2003] and early control of their mRNA levels may represent a potential targets for the prevention of osteopenia. Although MMP-2 has a role in the degradation of gelatin, elastin, and fibronectin within the bone matrix, it was markedly upregulated by anabolic mechanical signals, and suppressed by disuse. However, it is also important to consider that MMP-2 is expressed by osteoblasts [Meikle et al., 1992; Okada et al., 1995] and important for bone mineralization [Itoh et al., 1998; Satoyoshi et al., 2001]. Thus, the up-regulation of MMP-2 by mechanical signals and its down-regulation by disuse may be associated primarily with anabolic events and emphasizes the importance of considering a gene's role within the integrative milieu of the *in vivo* environment.

In contrast to the activities of MMP-2, gene expression patterns of another member of the MMP family, MMP-9, failed to correlate with any other candidate gene and was largely unaffected by either the catabolic or anabolic stimulus. The basis for the independent relationship of these two MMP genes in mechanically mediated bone adaptation may lie with the different cell types (osteoblasts *vs.* osteoclasts) expressing the two molecules [Okada et al., 1995; Wucherpfennig et al., 1994], the dependency of MMP on post-translational modification, and/or the differential regulation of MMP inhibitors (TIMP).

IGF-1, a gene critical to osteoblast progenitor proliferation and regeneration [Canalis et al., 1993; Lean et al., 1996], fracture healing [Bostrom et al., 1999], and the orchestration of the anabolic response to biochemical agents [Bikle et al., 2002], did not - at least at the two time points considered - appear to play a major role in either decreases or increases in bone formation as stimulated by changes in mechanical signals. This finding is consistent with preliminary (unpublished) data from our laboratory which fail to show any changes in IGF-1 serum levels in the mice used for this study at either day 4 or 21. While this could easily be explained by suggesting that the two time points

considered in this study simply missed the altered IGF-1 activity much earlier [Kawata et al., 1998; Lean et al., 1995], it is also possible that bone adaptation to the mechanical signals used in this study is such a "subtle" process that the mRNA levels of some genes typically associated with bone formation are never substantially perturbed.

Nitric oxide is a ubiquitous molecule that has been proposed as a key regulator of bone remodeling, through influencing pathways of both bone resorption and formation [Watanuki et al., 2002; Rubin et al., 2003; Chole et al., 1998]. The inducible form of nitric oxide synthase (iNOS or NOS2) is capable of producing greater quantities of nitric oxide than the constitutive form (eNOS), and its absence has been shown to prevent the catabolic events associated with menopause or mechanical disuse [Watanuki et al., 2002; Cuzzocrea et al., 2003; van't Hof et al., 2000]. In this study, the moderate correlation with RANKL expression patterns, a factor critical for osteoclast differentiation, may preferentially emphasize the resorptive role of iNOS. The up-regulation of iNOS and RANKL as measured at 21d in the mechanically stimulated animals may at first appear counterintuitive, but is consistent with the increased generation of nitric oxide in mechanical stimulation systems *in vitro* [Pitsillides et al., 1995; Klein-Nulend et al., 1995]. Alternatively, both nuclear factor κB and its ligand RANKL are expressed by osteoblasts and RANKL up-regulation could also reflect an increase in osteoblast number (as indicated by the increase in mineralizing surface in vibrated mice), and orchestrate not only a bone formation response, but the coupling to a bone resorption response [Parfitt, 1994]. Perhaps, relationships such as those will help identify the cascade of genes that are critically involved in the bone remodeling process.

In summary, these data emphasize that the molecular events involved in mechanically mediated bone adaptation are both subtle and complex. Further, the similarity in expression patterns between many distinct genes responding to the catabolic and/or anabolic signals accentuates an intricate co-dependence of molecular events involved in bone's adaptation to mechanical signals. Detailed

temporal assays using functional genomics and validated by histological assessment will ultimately contribute to a more complete characterization of the mechanisms which control bone adaptation, perhaps improving our understanding of the etiology of osteoporosis, and help to identify unique molecular targets that could prevent this devastating disease.

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TABLES

Target Gene	Forward (5' to 3')	Reverse (5' to 3')	Size	Tm (°C)
		Genes Involved in Bone Formation		
BMP-2	AGGATTAGCAGGTCTTTGC	GCCACGATCCAGTCATT	215	58
CBFA1	AGCAGCACTCCATATCT	CTTCCGTCAGCGTCAA	179	56
Collagen (α1)	CTGGCAAGAATGGCGA	GAAGCCACGATGACCC	161	55
Integrin-b3	AGGATTACCGACCCTCT	ATCTTGCCGAAGTCGC	121	57
IGF-1	CGCTCTGCTTGCTCACC	CCCCTCGGTCCACACA	103	58
MMP2	AGGTGTGCCAAGGTGGA	GAAGGAAACGAGCGAAGG	108	58
Osteonectin	GCATCAAGGAGCAGGACA	CGGAACAGCCAACCATC	139	58
Osterix	CCCAGAGCAGAGCAACC	CAGAGAGAGCCCCCAGA	150	57
		Genes Involved in Bone Resorption		
Cathepsin-K	GAAAATTGTGACCGTGATAATG	CGTTGTTCTTATTCCGAGC	116	56
MMP9	TCTGGCACACGCCTTTC	GGCACCATTTGAGTTTCCA	124	58
NOS2	CAGCACAAAGGGCTCAAA	CTCTCTTGCGGACCATCTC	109	57
Osteopontin	CGTCCCTACAGTCGATG	GCTGCCCCTTTCCGTTG	226	57
RANKL	CTGGTCGGGCAATTCT	CCCAAAGTACGTCGCAT	139	55
		Housekeeping Gene		
GAPDH	ACCAACTGCTTAGCCC	CTTCCCGTTCAGCTCT	222	55-58

Table 1. Primer sequences, generated amplicon size, and melting temperature corresponding to the 13 candidate genes (and GAPDH, the 'housekeeping' gene) used for this study. They are segregated into those genes proposed to be involved in bone formation, and those important in regulating bone resorption.

		BFR/BS [mm/yr]	MS/BS [%]	MAR [μm/d]
Trabecular	*CTR*	129±38	33.6±5.8	1.0±0.3
	VIB	170±32*	39.5±4.5*	1.2±0.2
	DIS	58±33*	27.0±7.3*	0.6±0.2*
Periosteal	*CTR*	61±38	37.7±17.5	0.4±0.1
	VIB	118±91	47.1±24.2	0.6±0.2*
	DIS	24±24*	14.1±10.2*	0.4±0.2
Endocortical	*CTR*	72±45	36.0±16.4	0.5±0.2
	VIB	117±77	48.0±18.0	0.6±0.2
	DIS	22±23*	18.3±12.0*	0.3±0.1*

Table 2. Bone formation rates (BFR/BS), mineralizing surface (MS/BS), and mineral apposition rates measured at trabecular (metaphysis) and cortical (middiaphysis) surfaces of control (CTR), vibrated (VIB), and disuse (DIS) mice (mean±SD). Asterisks indicate significant differences with respect to the control group.

		Cathepsin-K	Cbfa1	IGF-I	Integrin β3	MMP-9
4 days	*CTR*	3.43	2.18	2.37	1.52	1.58
	VIB	3.34 (-3%)	2.48 (14%)	2.34 (-1%)	1.44 (-5%)	1.80 (14%)
	DIS	3.49 (2%)	2.03 (-7%)	2.64 (12%)	1.56 (3%)	1.70 (8%)
21 days	*CTR*	3.35	2.31	2.40	1.67	1.91
	VIB	3.56 (6%)	2.45 (6%)	2.60 (8%)	1.79 (8%)	1.84 (-4%)
	DIS	3.53 (5%)	2.01 (-13%)	2.29 (-5%)	1.62 (-3%)	2.02 (6%)

Table 3. Expression ratios of genes (with GAPDH expression levels as referent) that were not significantly affected by either the anabolic low level mechanical vibration (VIB) or the catabolic signal, disuse (DIS) at either time point (4d and 21d), when compared to values measured in age-matched control mice (CTR). Percentages in parentheses represent the differences in mean expression ratios between the experimental and control groups.

Cluster 1	*MMP-2*	*CBFA1*	*Osteonectin*	*Osterix*	*BMP-2*	*Coll-I*
MMP-2	1.00					
CBFA1	0.67	1.00				
Osteonectin	0.78	0.68	1.00			
Osterix	0.78	0.83	0.86	1.00		
BMP-2	0.96	0.64	0.91	0.85	1.00	
Collagen-I	0.87	0.62	0.96	0.83	0.97	1.00

Table 4. Correlation coefficients between gene expression patterns across the experimental groups and time points. Grouping the genes based on the magnitude of their respective correlation coefficients, most genes that showed responsiveness to either vibration or disuse fell into the group above. Not surprisingly, genes within this cluster are functionally related (i.e., bone formation).

FIGURES

Figure 1. Mean (+SD) tibial bone formation rates (BFR/BS) of control, vibrated, and disuse mice averaged across the trabecular proximal metaphysis and the periosteal and endocortical surfaces of the middiaphysis. Asterisks indicate significant differences between the control and an experimental group ($p<0.05$).

Figure 2. mRNA levels of genes involved in *bone formation* normalized to mRNA levels of a housekeeping gene (GAPDH) in control (CTR), vibrated (VIB), and disuse (DIS) mice after 4d and 21d. Each graph presents the two mean values corresponding to the two pools of animals (Pool 1, Pool 2) as well as the average of these two means (Mean). Indicated percentages denote the statistically significant ($p<0.05$, unless noted) relative differences with respect to the mean expression value of the control group. Asterisks denote a significantly different ($p<0.05$) response between the two time points for a given gene and experimental condition.

Figure 3. mRNA levels of genes involved in *bone resorption* normalized to mRNA levels of a housekeeping gene (GAPDH) in control (CTR), vibrated (VIB), and disuse (DIS) mice after 4d and 21d. Each graph presents the two mean values corresponding to the two pools of animals (Pool 1, Pool 2) as well as the average of these two means (Mean). Indicated percentages denote the statistically significant ($p<0.05$, unless noted) relative differences with respect to the mean expression value of the control group. Asterisks denote a significantly different ($p<0.05$) response between the two time points for a given gene and experimental condition.

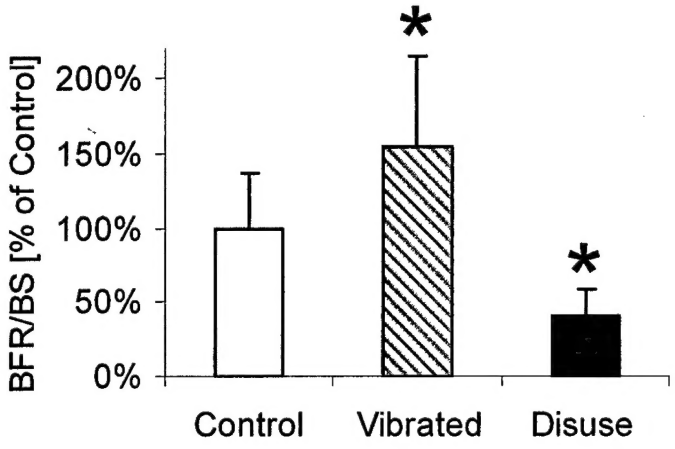

Figure 1. Mean (+SD) tibial bone formation rates (BFR/BS) of control, vibrated, and disuse mice averaged across the trabecular proximal metaphysis and the periosteal and endocortical surfaces of the middiaphysis. Asterisks indicate significant differences between the control and an experimental group ($p<0.05$).

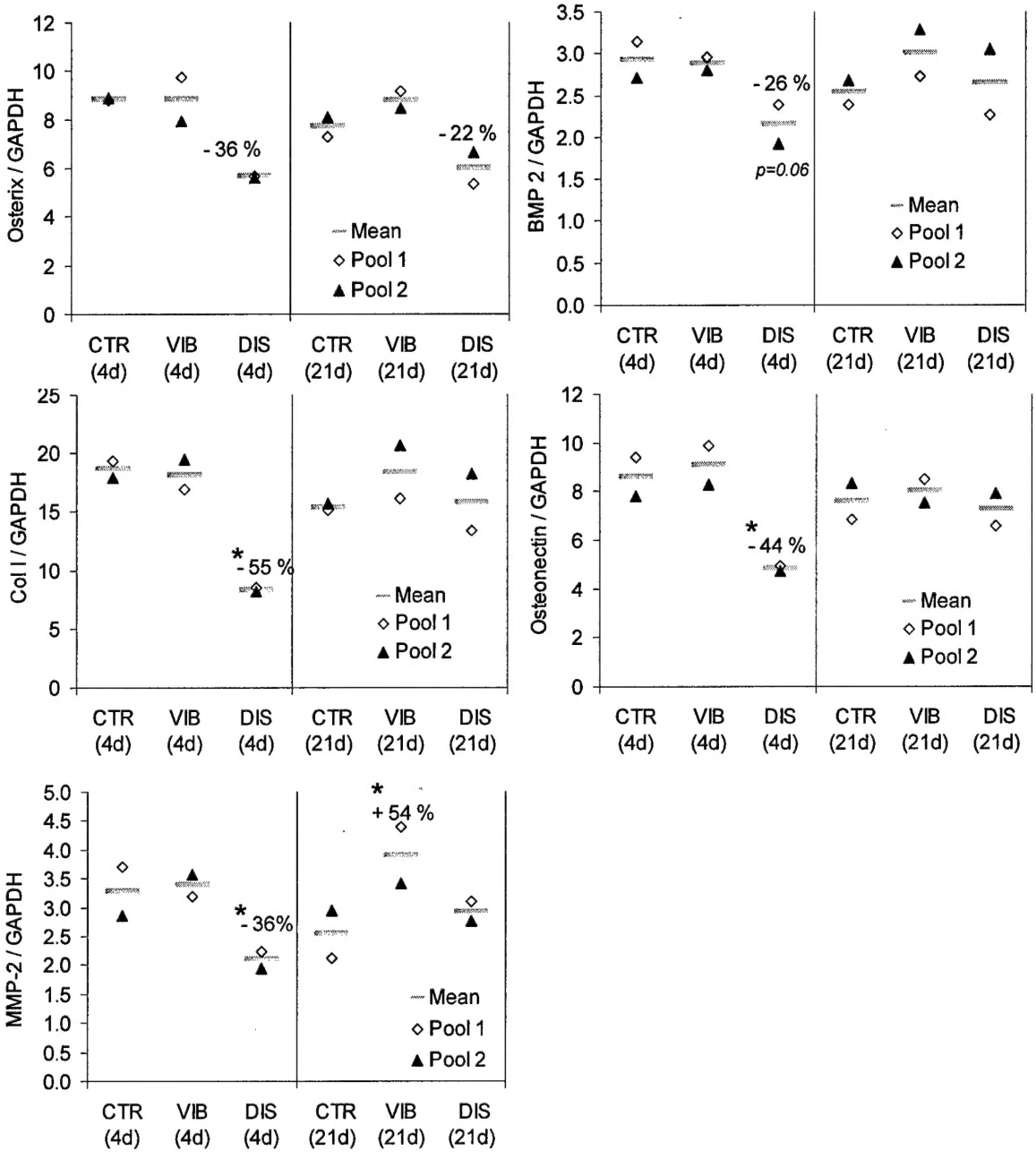

Figure 2. mRNA levels of genes involved in *bone formation* normalized to mRNA levels of a housekeeping gene (GAPDH) in control (CTR), vibrated (VIB), and disuse (DIS) mice after 4d and 21d. Each graph presents the two mean values corresponding to the two pools of animals (Pool 1, Pool 2) as well as the average of these two means (Mean). Indicated percentages denote the statistically significant ($p \leq 0.05$, unless noted) relative differences with respect to the mean expression value of the control group. Asterisks denote a significantly different ($p < 0.05$) response between the two time points for a given gene and experimental condition.

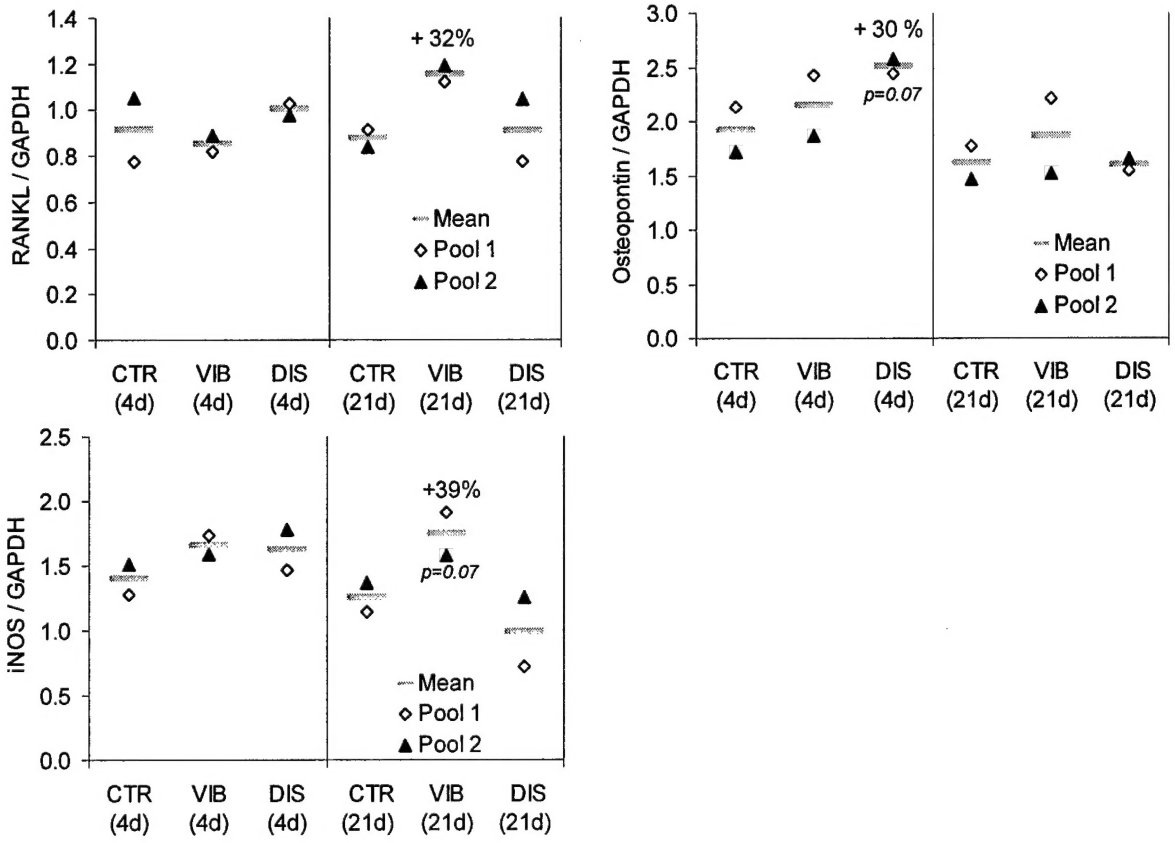

Figure 3. mRNA levels of genes involved in *bone resorption* normalized to mRNA levels of a housekeeping gene (GAPDH) in control (CTR), vibrated (VIB), and disuse (DIS) mice after 4d and 21d. Each graph presents the two mean values corresponding to the two pools of animals (Pool 1, Pool 2) as well as the average of these two means (Mean). Indicated percentages denote the statistically significant ($p<0.05$, unless noted) relative differences with respect to the mean expression value of the control group. Asterisks denote a significantly different ($p\leq0.05$) response between the two time points for a given gene and experimental condition.

High-frequency low-magnitude vibrations may induce vascular remodeling in skeletal muscle.

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Extremely low-magnitude mechanical signals, applied non-invasively as whole body vibrations, may stimulate bone formation. The objective of this study was to assess the effect of these potentially clinically relevant signals on microvascular remodeling in the mouse soleus muscle. Eight week old male BALB/cByJ (BALB) mice were divided into four experimental groups (n=6 each): 8-week baseline control, aged-matched control, 45 Hz (0.3g) vibration for 15 min/d, and 45 Hz (0.3g) vibration interrupted every 15 sec by 15 sec for 30 min/d. After the 6-week experimental protocol, sections from the end and mid regions of the soleus muscles were analyzed histologically and stained for BSI lectin and smooth muscle α-actin. Six weeks of normal growth caused a significant increase (52%) in capillary per fiber ratio (C/F) in the end region of the soleus muscle, but this response was inhibited by continuous vibration and vibration interrupted stimuli. C/F in the mid region and actin positive vessels per muscle fiber in both regions were not significantly different between experimental groups. Muscle fibers per area remained unchanged between experimental groups suggesting that the end region vascular remodeling is independent of changes in muscle fiber size. The decrease in C/F with vibrations may be associated with fiber type remodeling or microenvironment alterations induced by the low-level mechanical signals. These results suggest that vibrations applied at very low magnitudes may not only be capable of promoting bone formation but may also alter the vasculature of skeletal muscle.